



# TRITHORAX-dependent arginine methylation of HSP68 mediates circadian repression by PERIOD in the monarch butterfly

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**Transcriptional repression drives feedback loops that are central to the generation of circadian (~24-h) rhythms. In mammals, circadian repression of circadian locomotor output cycles kaput, and brain and muscle ARNT-like 1 (CLOCK:BMAL1)-mediated transcription is provided by a complex formed by PERIOD (PER) and CRYPTOCHROME (CRY) proteins. PER initiates transcriptional repression by binding CLK:BMAL1, which ultimately results in their removal from DNA. Although PER's ability to repress transcription is widely recognized, how PER binding triggers repression by removing CLK:BMAL1 from DNA is not known. Here, we use the monarch butterfly as a model system to address this problem because it harbors a simplified version of the CLK:BMAL1-activated circadian clock present in mammals. We report that an intact CLOCK mouse exon 19 homologous region (CLKe19r) and the histone methyltransferase TRITHORAX (TRX) are both necessary for monarch CLK:BMAL1-mediated transcriptional activation, CLK-PER interaction, and PER repression. Our results show that TRX catalytic activity is essential for CLK-PER interaction and PER repression via the methylation of a single arginine methylation site (R45) on heat shock protein 68 (HSP68). Our study reveals TRX and HSP68 as essential links between circadian activation and PER-mediated repression and suggests a potential conserved clock function for HSPs in eukaryotes.**

PERIOD | heat shock protein | arginine methylation | TRITHORAX | insect

**B**iological processes at all levels of organization and across taxa exhibit endogenous oscillations of about 24 h (i.e., circadian) that are synchronized by environmental cues to ensure optimal activity of biological functions at the appropriate time of day. In animals, circadian rhythms are tightly controlled by an internal cell-autonomous timing mechanism, the circadian clock, which consists of two interconnected transcriptional negative feedback loops. In mammals, the core time-keeping loop relies on the binding of the heterodimeric transcription factor CLOCK:BMAL1 (circadian locomotor output cycles kaput, and brain and muscle ARNT-like 1, respectively) to E-box elements during the day to activate the transcription of the circadian repressors *Period1/Period2* (*Per1/Per2*) and *Cryptochrome1/Cryptochrome2* (*Cry1/Cry2*). Upon accumulation, PERs and CRYs form a complex that translocates to the nucleus to repress CLOCK:BMAL1 activity at night. Subsequent PER-CRY degradation in the early morning allows CLOCK:BMAL1 to bind E boxes again and start transcription anew. While CRY1 acts as the dominant repressor through its interaction with BMAL1 C-terminal transactivation domain (TAD) (1–3), the binding of PER to CLOCK is necessary to initiate repression by the PER-CRY complex (4). The mechanism underlying PER recruitment to CLOCK:BMAL1 to initiate circadian repression remains, however, poorly understood.

Based on previous studies, one intriguing possibility is that repression by PERs and CRY1 may have converged onto activation domains of CLOCK and BMAL1, respectively (reviewed in ref. 5). In mammals, a domain located on exon 19 of CLOCK is

necessary for locomotor activity rhythms (6–8), circadian activation (9), and the rhythmic binding of the histone methyltransferase mixed lineage leukemia 1 (MLL1) (10). MLL1 is thought to establish a permissive chromatin state for circadian transcription by depositing histone 3 lysine 4 trimethylation (H3K4me3) marks at clock and clock-controlled gene promoters during CLOCK:BMAL1 activation (10). Interestingly, PER binds to mouse CLOCK exon 19 and to a conserved homologous region in *Drosophila* CLK (11), suggesting a possible function for CLOCK exon 19 in PER repression. This possibility is further supported by findings from luciferase assays in *Drosophila* Schneider 2 (S2) cells with clock proteins from the silkworm *Antheraea pernyi* that showed that PER-mediated repression of CLK:BMAL1 is lost in the absence of the CLK C terminus, which contains a conserved CLK exon 19 homologous region (12). The lepidopteran clock core feedback loop, most extensively studied in the monarch butterfly *Danaus plexippus*, shares striking similarities to that of mammals. In the monarch, circadian activation is provided by CLK:BMAL1, and circadian repression is mediated by PER and a mammalian-like CRY (designated dpCRY2) (13, 14) that functions, as in mammals, as the main transcriptional repressor through binding to the BMAL1 C-terminal TAD (1, 3, 15). This apparent functional analogy, together with the fact that the monarch clock possesses single

## Significance

**Circadian repression drives the transcriptional feedback loops that keep circadian (~24-h) time and synchronize an animal's physiology and behavior to the daily environmental changes. Although PERIOD (PER) is known to initiate transcriptional repression by displacing the transcription activator CLOCK:BMAL1 from DNA, the underlying mechanism remains unknown. Using the monarch butterfly as a model harboring a simplified version of the mammalian circadian clock, we demonstrate that the binding of heat shock protein 68 (HSP68) to a region homologous to CLOCK mouse exon 19 is essential for CLK-PER interaction and PER repression. We further show that CLK-PER interaction and PER repression are promoted by the methylation of a single arginine methylation site (R45) on HSP68 via TRITHORAX catalytic activity.**

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The authors declare no competing interest.

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copies of PER and mammalian-like CRY, makes the monarch butterfly an attractive model system for mechanistic studies of circadian repression by PER.

In the present study, we demonstrate that, similar to mammals, an intact CLK mouse exon 19 homologous region (CLKe19r) is required for circadian transcriptional activity and behavioral circadian rhythms *in vivo* in the monarch butterfly. We show that CLKe19r and the histone methyltransferase TRITHORAX (TRX), the invertebrate homolog of MLL1, are both necessary for monarch CLK:BMAL1-mediated transcriptional activation, CLK–PER interaction, and PER repression in *Drosophila* S2 cells. Unexpectedly, we found that the catalytic activity of TRX is essential for PER repression and formation of the CLK–PER complex via the direct or indirect methylation of a single arginine methylation site (R45) on heat shock protein 68 (HSP68). Based on the functional conservation of CLKe19r between species, we speculate that this mechanism may be conserved in mammals.

## Results

**CLK Exon 19 Homologous Region Is Necessary for CLK:BMAL1-Mediated Transcriptional Activation and Repression by PER.** To assess whether the monarch butterfly would be a relevant model to test if PER repression occurs through the homologous region of CLK exon 19 (CLKe19r), we started by testing whether this domain was necessary for circadian activation and behavioral rhythms in monarch butterflies, as is the case in mice (6–9). Using CRISPR-Cas9-mediated targeted mutagenesis, we generated a monarch mutant bearing a 287-bp deletion that eliminated the entire conserved CLK exon 19r located on monarch CLK exon 12 (thereafter called CLK $\Delta$ 19r) (Fig. 1 *A* and *B*). To measure circadian behavior, we used pupal eclosion as our behavioral readout because no assays have been developed to measure monarch locomotor activity. Similar to that reported in mice (7), monarch CLK $\Delta$ 19r hemizygous female mutants exhibited a long-period phenotype in circadian eclosion behavior when compared with wild-type sibling female controls (Fig. 1*C*). Moreover, circadian eclosion of CLK $\Delta$ 19r heterozygous male mutants was also lengthened but to intermediate levels compared with hemizygous female mutants (Fig. 1*C*), indicative of a semidominant function for the mutant allele as previously shown in mice (8). CLK $\Delta$ 19r hemizygous female mutants also showed disrupted molecular rhythms in the brain with constitutive low expression levels of *period* (*per*) and *timeless* (*tim*) messenger RNA (mRNA) under constant conditions (Fig. 1*D*). Together, these results show that monarch CLK $\Delta$ 19r mutants are defective in circadian activation and phenocopy CLK $\Delta$ 19 mutant mice.

To determine whether CLKe19r is necessary for PER repression, we next used luciferase reporter gene assays in *Drosophila* S2 cells in which monarch proteins were coexpressed with a reporter construct containing a tandem repeat of the proximal CACGTG E-box enhancer from the monarch *per* gene promoter (14). As previously reported (13, 14), cotransfections of the luciferase reporter with monarch CLK and BMAL1 increased substantially transcriptional activity, and monarch PER inhibited CLK:BMAL1-mediated transcription in a dose-dependent manner (Fig. 2*A*). Consistent with our *in vivo* analysis of the CLK $\Delta$ 19r mutant, we also found that CLK $\Delta$ 19r:BMAL1-mediated transcription was severely reduced by about fivefold (Fig. 2*A*). Interestingly, repression of CLK $\Delta$ 19r:BMAL1-mediated transcription by PER was abolished (Fig. 2*A*). In contrast and consistent with its known repressive action on the BMAL1 C-terminal domain (15), CRY2 was able to repress CLK $\Delta$ 19r:BMAL1-mediated transcription, demonstrating that CLKe19r is necessary for PER repression but not for CRY2 repression (Fig. 2*A*). To bolster this finding, we performed luciferase assays in S2

cells using the CLKe19r domain as a sponge. We reasoned that if PER represses CLK:BMAL1 through its interaction with CLKe19r, coexpressing the CLKe19r domain should displace PER away from CLK:BMAL1, thereby releasing repression. In line with our hypothesis, we found that coexpressing increasing doses of the CLKe19r domain fused to the Gal4 DNA binding domain (Gal4DBD), used to ensure folding of the short ~50-amino acid CLKe19r, caused a dose-dependent inhibition of repression of CLK:BMAL1 by PER (Fig. 2 *B*, *Left*). Expressing high doses of Gal4DBD alone did not inhibit PER repression (Fig. 2 *B*, *Right*), showing that the effect of the sponge was solely due to the presence of CLKe19r.

We further tested whether CLKe19r would be sufficient for PER repression using a transactivation assay where a luciferase reporter gene under the control of a six tandem repeats of the yeast GAL4 upstream activator sequence (6X UAS) promoter (the DNA binding site of Gal4DBD) was cotransfected with either the strong herpes simplex virion protein 16 (VP16) TAD directly fused to Gal4DBD or the Gal4DBD-CLKe19r fusion protein (Fig. 2*C*). We found that Gal4DBD-VP16 and Gal4DBD-FLAG-CLKe19r-VP16 both potently activated transcription but that only Gal4DBD-FLAG-CLKe19r-VP16 was repressible by PER in a dose-dependent fashion (Fig. 2*C*), further demonstrating that CLKe19r is sufficient for PER repression. We noted that in the absence of VP16, Gal4DBD-FLAG-CLKe19r did not activate transcription (*SI Appendix*, Fig. S1*A*), suggesting that CLKe19r is necessary, but not sufficient, for transcriptional activation; thus, transactivation requires other domains on CLK.

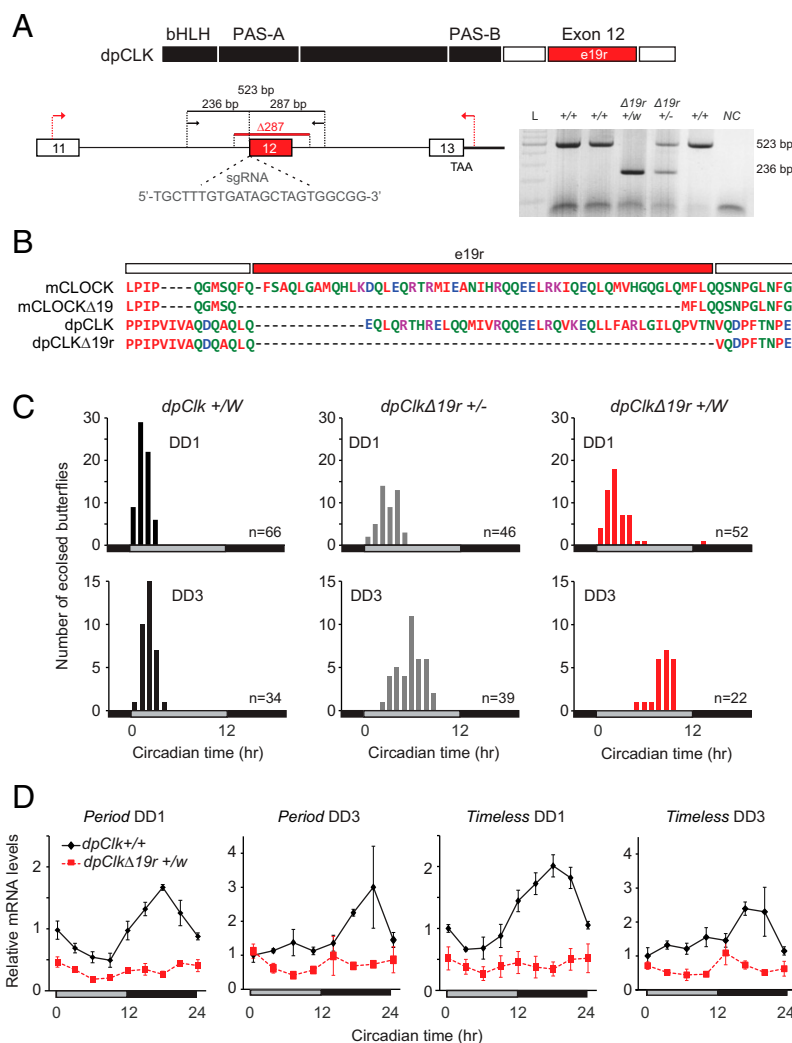
## The Histone Methyltransferase TRX Is Necessary for CLK:BMAL1-Mediated Transcriptional Activation and Repression by PER.

Because CLKe19r is necessary for the rhythmic binding of the histone methyltransferase MLL1 that deposits H3K4me3 marks at core clock gene promoters during CLOCK:BMAL1 activation in mammals (10), we tested whether its insect homolog, TRX (16), was also necessary for both CLK:BMAL1 activation and PER repression. To this end, we coexpressed CLK and BMAL1 in the absence or presence of increasing doses of PER in S2 cells in which endogenous *Trx* was knocked down by RNA interference (RNAi). RNAi-mediated knockdown of endogenous *Trx* caused a significant decrease in CLK:BMAL1 activation and eliminated repression by PER but not by CRY2 (Fig. 2*D* and *SI Appendix*, Fig. S1*B*), similar to that observed with CLK $\Delta$ 19r:BMAL1 (Fig. 2*A*). These results suggest that both CLKe19r and TRX are required not only for CLK:BMAL1-mediated transcriptional activation but also, for PER repression. We confirmed that TRX action occurs through the CLKe19r domain by showing that Gal4DBD-FLAG-CLKe19r-VP16-mediated transcriptional activation is severely blunted, and its repression by PER is abolished when *Trx* is knocked down (*SI Appendix*, Fig. S1*C*).

To test whether CLKe19r and TRX were also necessary for CLK–PER interaction, we performed coimmunoprecipitations (co-IPs) of FLAG-tagged PER coexpressed in S2 cells with either V5-tagged CLK $\Delta$ 19r or V5-tagged CLK in the presence or absence of endogenous *Trx*. While PER and CLK interacted in the presence of TRX, we found that the interaction was abolished in the absence of CLKe19r or TRX (Fig. 2*E*). Taken together, our results demonstrate that both CLKe19r and TRX are required not only for CLK:BMAL1-mediated transcriptional activation and repression by PER but also, for CLK–PER interaction.

## The Catalytic Activity of TRX Mediates PER Repression and CLK–PER Interaction.

Based on the previously reported binding of MLL1 to CLOCK exon 19 in mammals (10), our results suggested that TRX could function as a scaffold protein for CLK–PER



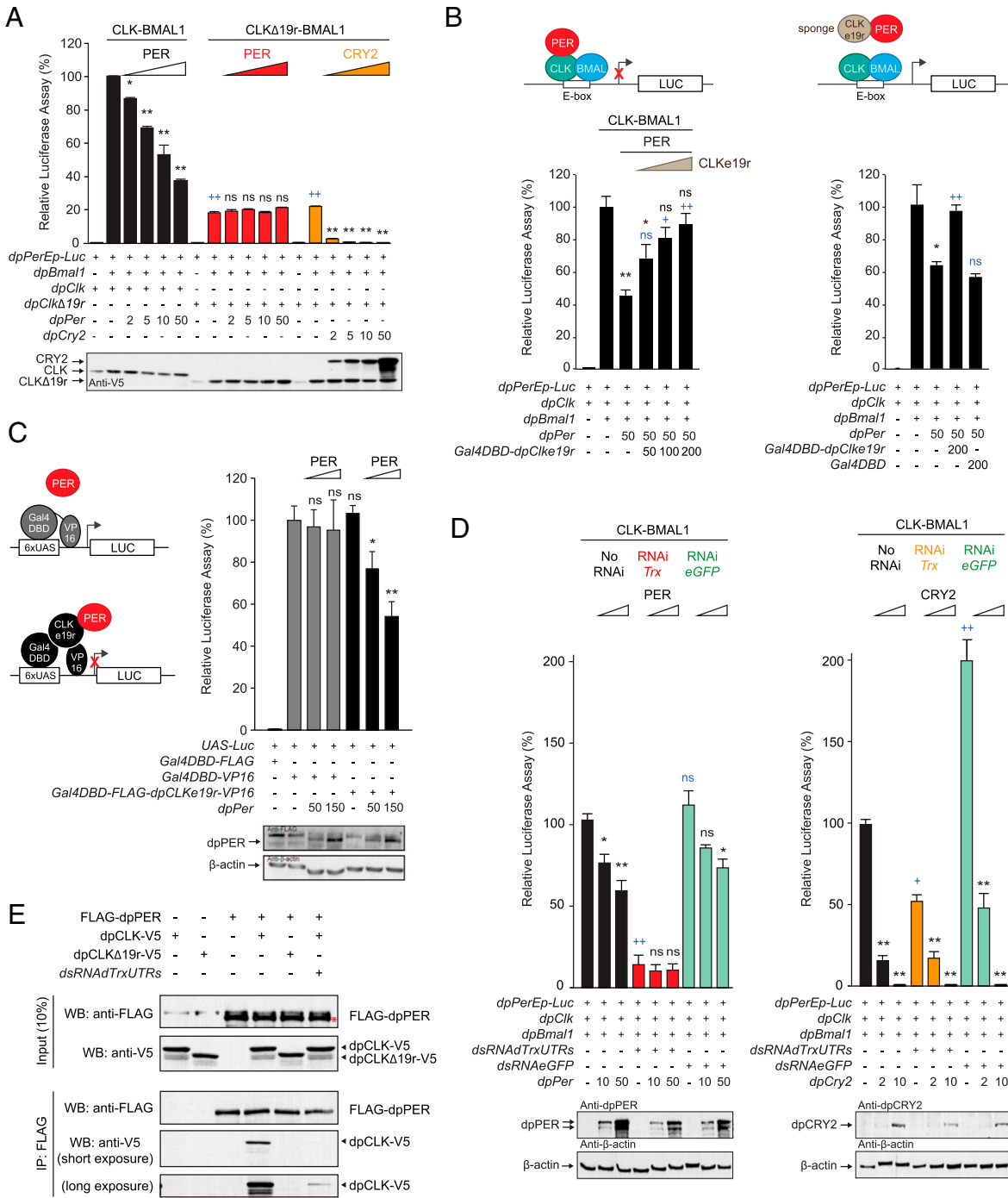
**Fig. 1.** Monarch circadian rhythms and transcriptional activity are dependent on an intact mouse exon 19 homologous region (e19r) on CLK. (A) Depiction of monarch (dp) CLK and its e19r located on exon 12 (Upper). bHLH, basic Helix–Loop–Helix; PAS, Per–Arnt–Sim. Position of the single-guide RNA (sgRNA) used to generate a CRISPR–Cas9–mediated 287-bp deletion containing exon 12 and primers (black arrows) used to amplify the 523-bp targeted region for analysis of mutagenic lesions (Lower Left). PCR-based detection of mutagenic lesions ( $\Delta 19r$ ) in somatic cells of G2 butterflies (Lower Right). L, ladder; NC, no template control. (B) Sequences alignment showing conservation of the CLKe19r region in mouse (m) and monarch (dp) CLOCK proteins and corresponding  $\Delta 19r$  deletion mutants. (C) Profiles of adult eclosion during the first or third day of constant darkness (DD1 and DD3, respectively) after entrainment to 12-h light: 12-h dark in wild-type (black), *dpClk $\Delta 19r$*  heterozygous (gray), and heterozygous mutant (red) siblings. Data are binned in 1-h intervals. Horizontal bars show subjective day (gray) and night (black). For each DD1 and DD3 condition, one-way ANOVA:  $P < 0.0001$ ; Tukey post hoc test: *dpClk $^{+/W}$*  vs. *dpClk $\Delta 19r$  $^{+/-}$* ,  $P < 0.01$ ; *dpClk $^{+/W}$*  vs. *dpClk $\Delta 19r$  $^{+/W}$* ,  $P < 0.01$ . (D) Relative mRNA expression levels of *per* and *tim* in brains of wild-type (black) and *dpClk $\Delta 19r$*  heterozygous mutants (red) over 24 h in DD1 and DD3. Data are represented as mean  $\pm$  SEM of three animals, except for the wild type at DD3 where mean  $\pm$  SEM is of four animals. Two-way ANOVA, interaction genotype  $\times$  time: *per* DD1,  $P < 0.05$ ; *tim* DD1,  $P < 0.0005$ ; *per* DD3,  $P = 0.059$ ; *tim* DD3,  $P = 0.078$ .

interaction. Full MLL1 catalytic activity on H3K4 methylation occurs through the assembly of its cleaved N-terminal and catalytically active C-terminal SET (Su(var)3-9, Enhancer-of-zeste, Trithorax) domains to a WRAD multiprotein complex composed of WD repeat-containing protein 5 (WDR5), retinoblastoma-binding protein 5 (RbBP5), absent, small, or homeotic discs 2-like protein (Ash2L), and dumpy-30 (Dpy-30) (17, 18). Importantly, WDR5 was previously shown to function as a modulator of PER activity that helps PER repression of CLOCK:BMAL1 (19). We thus examined whether TRX catalytic activity could play a role in PER repression of CLK:BMAL1-mediated transcription. Similar to mammalian MLL1, TRX possesses a cleavage site for the production of N-terminal and C-terminal subunits, and the SET domain is located in the C-terminal subunit (Fig. 3A).

We first tested whether the SET domain of TRX was necessary for PER repression by cotransfecting S2 cells in which

endogenous *Trx* was knocked down with CLK, BMAL1, increasing doses of PER, the N-terminal domain of TRX (TRX-NT), and either the wild-type C-terminal domain of TRX (TRX-CT) or a truncated version lacking the SET domain (TRX-CT $\Delta$ SET). Knockdown of endogenous *Trx* was achieved by using double-stranded RNAs (dsRNAs) targeting the 5' and 3' untranslated regions, which are absent on the TRX variants containing plasmids. Strikingly, we found that while coexpression of TRX-NT and TRX-CT restored both CLK:BMAL1 activation and its repression by PER, coexpressing TRX-NT and TRX-CT $\Delta$ SET restored activation levels but not PER repression (Fig. 3B). Furthermore, coexpressing TRX-NT in these experimental conditions marginally contributed to the restoration of activation to wild-type levels but was dispensable for PER repression (SI Appendix, Fig. S2A).

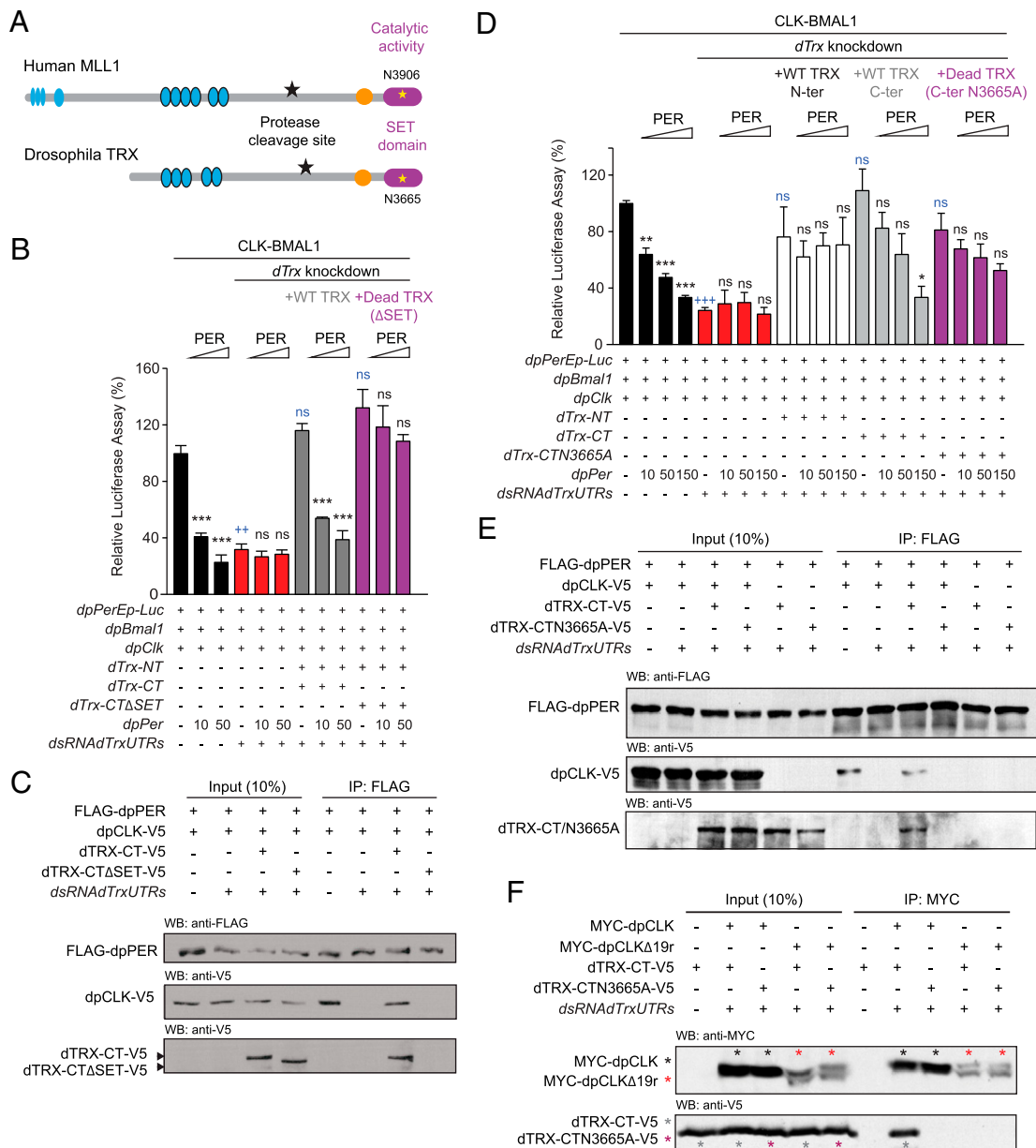
Because the SET domain of TRX appeared to be necessary for PER repression of CLK:BMAL1-mediated transcription,



**Fig. 2.** CLK $\Delta$ 19r and the histone methyltransferase TRX are necessary for CLK:BMAL1-mediated transcriptional activation, PER repression, and CLK-PER interaction. (A, Upper) The monarch *per* E-box luciferase reporter (dpPerEp-Luc; 10 ng) was expressed in *Drosophila* S2 cells in the presence of a dpBMAL1 expression plasmid and either dpCLK or dpCLK $\Delta$ 19r (5 ng each), with increasing doses of dpPER or dpCRY2 (amounts are given in nanograms). Firefly luciferase activity was computed relative to renilla luciferase activity. Each value is mean  $\pm$  SEM of three replicates. For each condition, one-way ANOVA, Tukey post hoc: \* $P$  < 0.05, \*\* $P$  < 0.01, and ns is nonsignificant (in black for repression); \* $P$  < 0.05, \*\* $P$  < 0.01, and ns is nonsignificant (in blue for activation). (A, Lower) Western blots of V5-tagged dpCLK, dpCLK $\Delta$ 19r, and dpCRY2. +, presence; -, absence. (B) dpPerEp-Luc (10 ng) was used in the presence of dpCLK, dpBMAL1 (5 ng each) and dpPER, without or with either Gal4DBD or Gal4DBD-dpCLK $\Delta$ 19r (amounts are given in nanograms). Quantification of luciferase activity and statistics were performed as in A. (C) A UAS-driven luciferase reporter (UAS-Luc; 10 ng) was used in the presence or absence of plasmids expressing Gal4DBD-FLAG or dpCLK $\Delta$ 19r fused to an N-terminal Gal4DBD-FLAG and a C-terminal VP16 transactivation domain (Gal4DBD-FLAG-dpCLK $\Delta$ 19r-VP16; 5 ng each) and increasing doses of dpPER (amount is given in nanograms). Quantification of luciferase activity and statistics were performed as in A. Western blots were performed using the indicated antibodies. (D) dpPerEp-Luc (10 ng) was used in the presence of dpCLK and dpBMAL1 expression plasmids (5 ng each) with increasing doses of dpPER or dpCRY2 (amounts are given in nanograms) and in the presence or absence of dsRNA against *egfp* or the 5' and 3' untranslated regions (UTRs) of *dTrx* (7.5  $\mu$ g each). Quantification of luciferase activity and statistics were performed as in A. Western blots were performed using the indicated antibodies. (E) Co-IPs from S2 cells transfected with FLAG-dpPER and either dpCLK $\Delta$ 19r-V5- or dpCLK-V5-expressing plasmids (500 ng each) in the presence or absence of dsRNA against *dTrx* 5' and 3' UTRs (7.5  $\mu$ g each). Co-IPs were probed with an anti-FLAG antibody, and western blots (WBs) were performed using the indicated antibodies. Red asterisk, dpPER protein. IP, immunoprecipitation.

we examined whether this domain could also be important for CLK-PER interaction. Co-IPs of FLAG-tagged PER coexpressed with V5-tagged CLK alone or in combination with

either V5-tagged TRX-CT or TRX-CT $\Delta$ SET in S2 cells in which endogenous *Trx* was knocked down revealed that CLK and TRX-CT were pulled down with PER only in the presence



**Fig. 3.** The catalytic activity of TRX is required for PER repression of CLK:BMAL1 and for CLK-PER interaction. (A) Schematic depicting conservation of the SET domains (purple boxes) and catalytic residues of human MLL1 (N3906) and its *Drosophila* ortholog TRX (N3665; yellow stars). Black stars indicate protease cleavage sites; colored ovals indicate other functional domains. (B) The monarch *per* E box luciferase reporter (dpPerEp-Luc; 10 ng) was expressed in S2 cells in the presence of dpBMAL1 and dpCLK expression plasmids (5 ng each) with increasing doses of dpPER (amounts are given in nanograms), in the absence of dsRNA against endogenous *dTrx* untranslated regions (UTRs), or in its presence along with the N-terminal (NT) fragment of dTRX and either the wild-type or a catalytically dead C-terminal (CT) fragment of dTRX lacking the SET domain (50 ng each). Firefly luciferase activity was computed relative to renilla luciferase activity. Each value is mean  $\pm$  SEM of three replicates. For each set of conditions: one-way ANOVA, Tukey post hoc: \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , and ns is nonsignificant (in black for repression); +\* $P < 0.01$ , and ns is nonsignificant (in blue for activation). (C) Co-IPs from S2 cells transfected with FLAG-dpPER, dpCLK-V5 expressing plasmids (150 ng each) without or with dsRNA targeting *dTrx* 5' and 3' UTRs (7.5  $\mu$ g each) and plasmids expressing either the wild-type dTRX-CT or catalytically inactive (dead) dTRX-CT $\Delta$ SET (150 ng each). Co-IPs were probed with an anti-FLAG antibody, and western blots (WBs) were performed using the indicated antibodies. (D) dpPerEp-Luc (10 ng) was used in the presence of dpBMAL1 and dpCLK expression plasmids (5 ng each) with increasing doses of dpPER (amounts are given in nanograms) without or with dsRNA targeting endogenous *dTrx* and plasmids expressing dTRX-NT, the wild-type dTRX-CT, or the mutant dTRX-CTN3665A (50 ng each). Quantification of luciferase activity and statistics were performed as in B. +\* $P < 0.001$ . (E) Co-IPs from S2 cells transfected with plasmids expressing FLAG-dpPER and dpCLK-V5 (150 ng each) without or with dsRNA targeting *dTrx* 5' and 3' UTRs (7.5  $\mu$ g each) and plasmids expressing either the wild-type or N3665A mutant dTRX-CT (150 ng each). Co-IPs were probed with an anti-FLAG antibody, and WBs were performed using the indicated antibodies. (F) Co-IPs from S2 cells transfected with plasmids expressing MYC-tagged dpCLK or dpCLK $\Delta$ 19r (200 ng each), dsRNA targeting *dTrx* 5' and 3' UTRs (7.5  $\mu$ g each), and plasmids expressing either the wild-type or N3665A mutant dTRX-CT (500 ng each). Co-IPs were probed with an anti-MYC antibody, and WBs were performed using the indicated antibodies. Asterisks: black, dpCLK; red, dpCLK $\Delta$ 19r; gray, dTRX-CT; purple, dTRX-CTN3665A. IP, immunoprecipitation.

of the SET domain on TRX-CT (Fig. 3C). These results suggested that the catalytic domain and/or activity of TRX was necessary for both CLK–PER interaction and PER repression.

To distinguish between these two possibilities, we generated a catalytically inactive TRX and tested its involvement in CLK:BMAL1-mediated transcription, PER repression, and CLK–PER interaction. Asparagine 3906 within the SET domain of human MLL1, which is conserved and located at position 3,665 of *Drosophila* TRX (Fig. 3A), was previously shown to be required for binding of the methyl donor *S*-adenosyl methionine but not for the overall structure of MLL1 (18). To generate catalytically inactive TRX, asparagine 3665 of TRX-CT was mutated to alanine to form TRX-CTN3665A. We performed luciferase reporter assays in S2 cells cotransfected with CLK, BMAL1, increasing doses of PER, RNAi against endogenous *Trx*, and either TRX-NT, TRX-CT, or TRX-CTN3665A. Results showed that, similar to what we found with TRX-CTΔSET, TRX-CTN3665A restored most CLK:BMAL1 transcriptional activation but failed to rescue PER repression, even at high doses of *per*, as compared with control TRX-CT (Fig. 3D and *SI Appendix*, Fig. S2 B and C). In addition, co-IPs of FLAG-tagged PER coexpressed with V5-tagged CLK alone or in combination with V5-tagged TRX-CT or V5-tagged TRX-CTN3665A in cells in which endogenous *Trx* was knocked down showed that the PER–CLK–TRX interaction was maintained with TRX-CT but abolished with the TRX-CTN3665A mutant (Fig. 3E). We verified that the lack of PER–CLK–TRX interaction with the TRX-CTN3665A mutant was not due to TRX-CTN3665A misfolding by showing that both TRX-CT and TRX-CTN3665A were able to interact with the SET domain of a previously described interactor, the absent, small, and homeotic-1 (ASH1) protein (20) (*SI Appendix*, Fig. S2D). Moreover, we showed that in the absence of CLK, PER failed to interact with TRX-CT, regardless of whether it is catalytically active or inactive, suggesting that the formation of the complex requires CLK (Fig. 3E).

To test whether TRX-CT interacts with CLK through CLKe19r as in mammals, we then performed co-IPs of either MYC-tagged CLK or CLKΔ19r, each coexpressed with either V5-tagged TRX-CT or V5-tagged TRX-CTN3665A. We found that the interaction between CLK and TRX-CT was abolished in the absence of CLKe19r or TRX catalytic activity (Fig. 3F), demonstrating that TRX interacts with CLK through CLKe19r and that the catalytic activity of TRX is required for the CLK–TRX interaction. Altogether, these results demonstrate that TRX promotes CLK–PER interaction and PER repression via its methyltransferase catalytic function rather than as a scaffold protein.

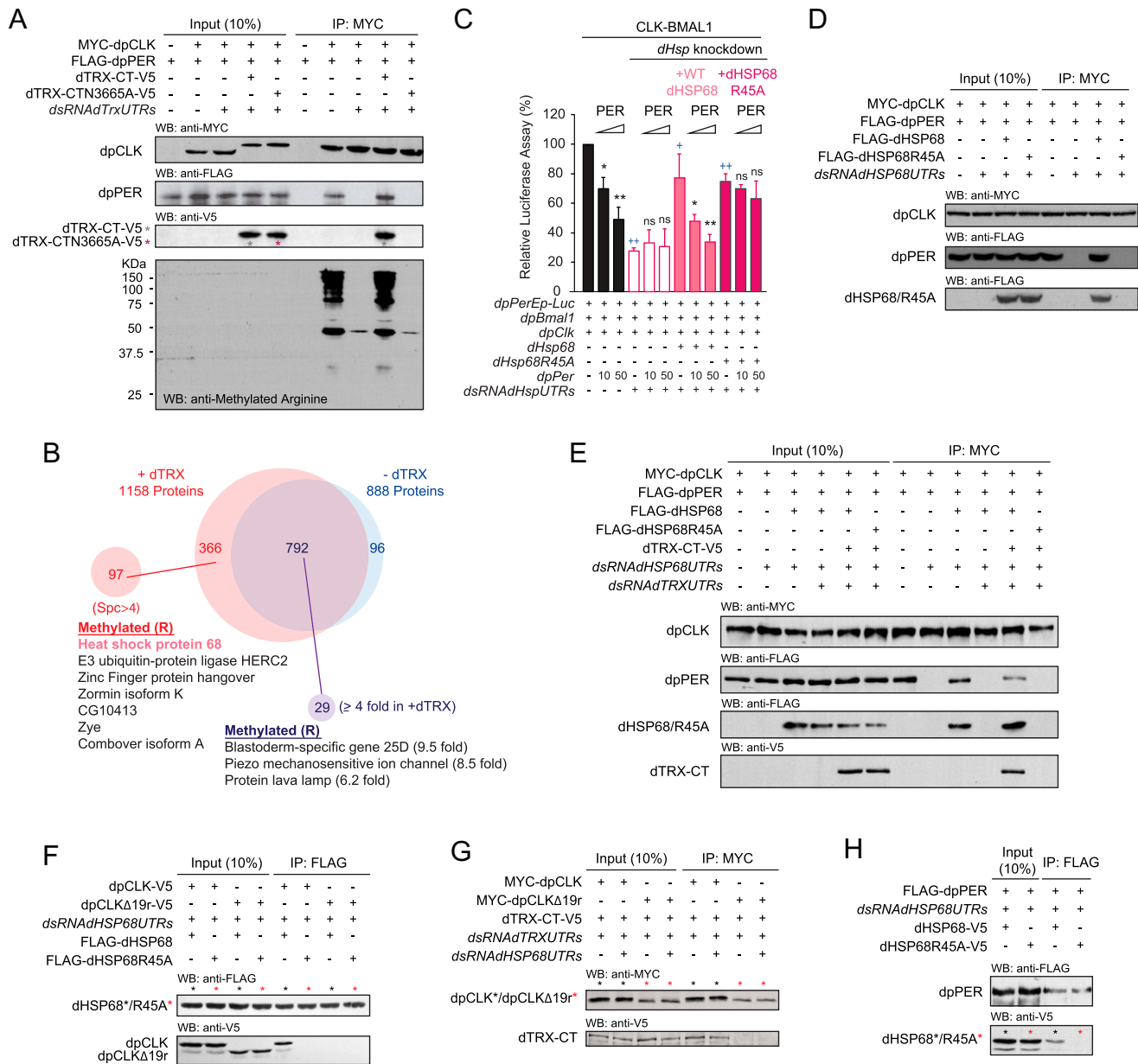
**TRX-Dependent Arginine Methylation of HSP68 Is Necessary for PER Repression and for CLK–PER Interaction.** Given the established catalytic role of TRX in monomethylating lysine 4 of histone 3 (21), we hypothesized that TRX may function in the methylation of yet unknown proteins on lysines that would be required for the CLK–TRX–PER interaction. To test this possibility, we repeated co-IPs from S2 cells cotransfected with MYC-tagged CLK and FLAG-tagged PER in the presence or absence of endogenous *Trx* with coexpressed V5-tagged TRX-CT or TRX-CTN3665A and immunoblotted for lysine-methylated proteins using a pan antimethylated lysine antibody. We predicted that lysine-methylated proteins would be present in conditions where CLK–PER interacts in the presence of endogenous TRX or exogenous TRX-CT but not when CLK–PER interaction is abolished in the absence of endogenous TRX or exogenous catalytically inactive TRX-CTN3665A. Unexpectedly, we found that lysine-methylated proteins were present in all conditions (*SI Appendix*, Fig. S3A), suggesting that the catalytic activity of TRX serves another function. Because protein methylation can occur

on lysine and arginine residues, we tested whether proteins were methylated on arginine by probing the pull down with a pan antimethylated arginine antibody. To our surprise and consistent with our original prediction, we found that arginine-methylated proteins pulled down only in the presence of endogenous TRX or exogenous TRX-CT (Fig. 4A). These results suggest that the histone methyltransferase TRX may function either as a protein arginine methyltransferase (PRMT) or in the recruitment of such an enzyme to methylate a protein necessary for the formation of the CLK–TRX–PER complex.

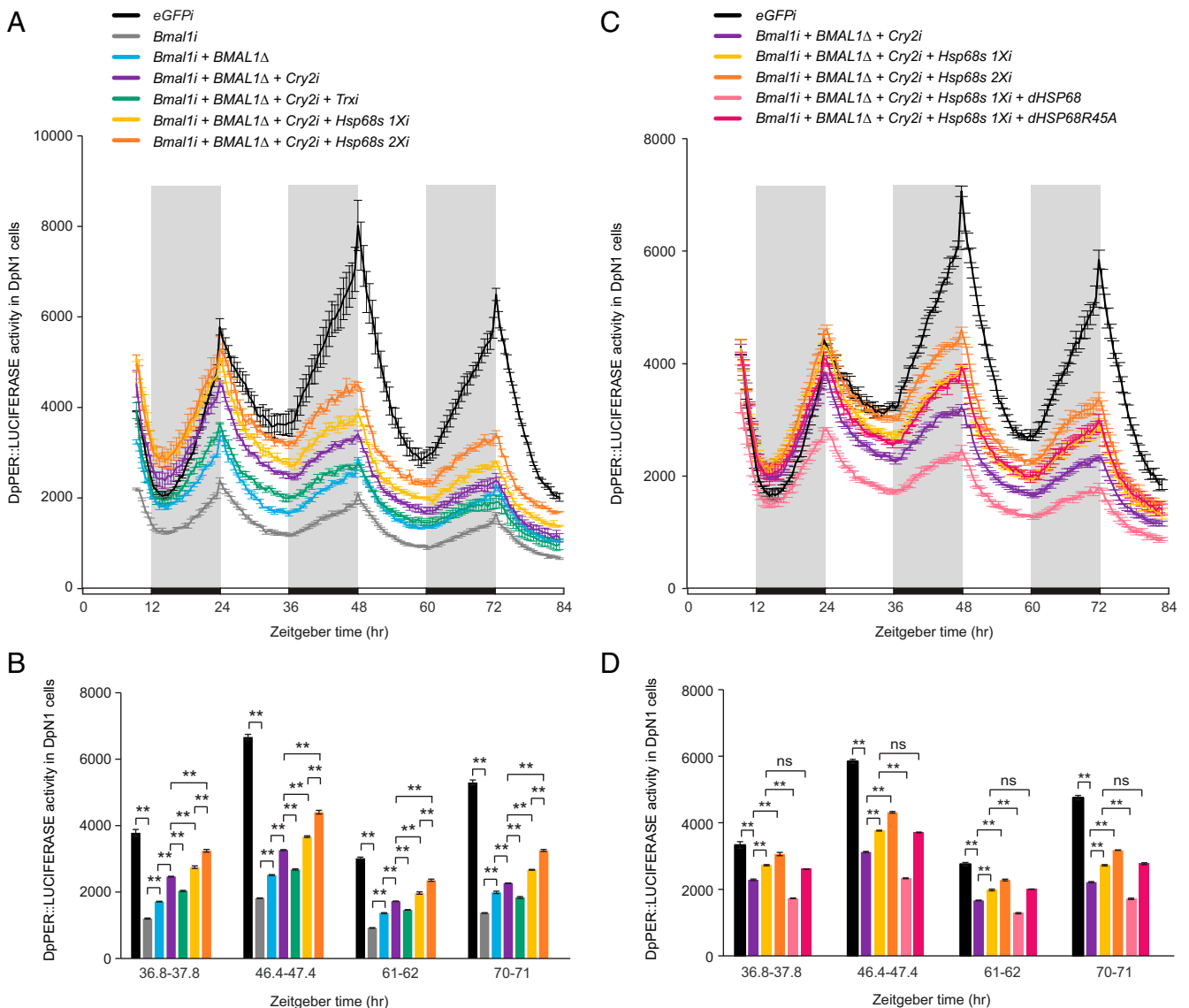
To identify candidate proteins methylated on arginine in the presence of TRX, we performed co-IPs in S2 cells cotransfected with MYC-tagged CLK and FLAG-tagged PER in the presence or absence of endogenous *Trx* (*SI Appendix*, Fig. S3B), followed by mass spectrometry. We identified 1,158 and 888 protein-unique peptides in the presence and absence of TRX, respectively, with 792 present in both conditions (Fig. 4B and *Dataset S1*). Among the 792 common to both conditions, 29 were enriched by fourfold or greater in the presence of TRX, of which 3 were methylated on arginine (Fig. 4B and *Dataset S1*). Of the 366 uniquely identified peptides in the presence of TRX, 7 of 97 protein-unique peptides with more than four spectral counts were also methylated on arginine (Fig. 4B and *Dataset S1*). We focused on the chaperone protein HSP68 (HSP of 68 kDa), which is methylated on a single arginine residue at position 45. Interestingly, methylation of another conserved arginine residue on its mammalian homolog HSP70 has recently been reported to regulate HSP70 function in transcription on chromatin (22).

We first assessed HSP68 function in CLK:BMAL1-mediated transcription and PER repression using luciferase reporter assays. Similar to what we found with *Trx* knockdown, CLK:BMAL1-mediated activation was significantly reduced and PER repression was abolished when endogenous *Hsp68* was knocked down (Fig. 4C and *SI Appendix*, Fig. S3C). Coexpressing exogenous wild-type HSP68 rescued both CLK:BMAL1 activation and PER repression. In contrast, coexpression of HSP68 bearing an alanine point mutant on R45 (HSP68R45A) rescued activation but not PER repression (Fig. 4C), demonstrating that methylation of HSP68 on R45 is required for repression of CLK:BMAL1 by PER but not for transcriptional activation. Using co-IPs, we also showed that, similar to what we found for TRX catalytic activity, methylation of HSP68 on R45 was necessary for CLK–PER interaction, as wild-type HSP68 but not HSP68R45A rescued the otherwise disrupted interaction when endogenous *Hsp68* was knocked down (Fig. 4D). To unambiguously demonstrate that TRX-dependent methylation of HSP68 on R45 is required for CLK–PER interaction, we performed similar co-IPs but in cells where both endogenous *Trx* and *Hsp68* were knocked down and cotransfected with V5-tagged TRX-CT and either FLAG-tagged wild-type HSP68 or the HSP68R45A mutant. We found that the CLK–HSP68–TRX–PER complex was reconstituted only in the presence of wild-type HSP68 (Fig. 4E).

We also tested the impact of methylation of HSP68 on R45 on the CLK–HSP68 interaction as well as the presence/absence of HSP68 for CLK–TRX binding in the absence of PER. We found that while HSP68 methylation on R45 was necessary for CLK–HSP68 interaction, HSP68 was dispensable for CLK–TRX binding (Fig. 4F and G), suggesting that TRX binds to CLK independently of HSP68. Both CLK–TRX and CLK–HSP68 interactions require, however, the presence of CLKe19r (Fig. 4F and G). Taken together, these results are consistent with the idea that TRX binding to CLK occurs through CLKe19r and that HSP68 binds to TRX. Furthermore, our findings that HSP68R45A is able to rescue to a great extent activation by CLK:BMAL1 in luciferase reporter assays (Fig. 4C) despite the lack of CLK–HSP68R45A interaction suggest that HSP68 likely plays two roles: one in



**Fig. 4.** TRX-dependent arginine methylation of HSP68 is necessary for PER repression and for CLK-PER interaction. (A) Co-IPs from S2 cells transfected with MYC-tagged dpCLK and FLAG-dpPER expressing plasmids (500 ng each) without or with dsRNA targeting *dTrx* 5' and 3' untranslated regions (UTRs) (15 μg each) and plasmids expressing either the wild-type or N3665A mutant *dTRX-CT* (500 ng each). Co-IPs were probed with an anti-MYC antibody, and western blots (WBs) were performed using the indicated antibodies, including an antimethylated arginine antibody. (B) Venn diagram of proteins identified using mass spectrometry that pulled down with MYC-dpCLK coexpressed with FLAG-dpPER in S2 cells in the presence or absence of endogenous dTRX. Among peptides pulled down in the presence (+) of TRX with spectral counts greater than four in each category (unique to +TRX or present in both conditions but enriched in +TRX), the ones methylated on arginine are listed. (C) The monarch *per* E box luciferase reporter (dpPerEp-Luc; 10 ng) was expressed in the presence of dpBMAL1 and dpCLK expression plasmids (5 ng each), increasing doses of dpPER (amounts are given in nanograms) without or with dsRNA targeting endogenous *dHsp68* 5' and 3' UTRs (7.5 μg each), and plasmids expressing either wild-type or R45A mutant dHSP68 (50 ng each). Firefly luciferase activity was computed relative to renilla luciferase activity. Each value is mean ± SEM of three replicates. For each panel, one-way ANOVA, Tukey post hoc: \**P* < 0.05, \*\**P* < 0.01, and ns is nonsignificant (in black for repression); +*P* < 0.05 and ++*P* < 0.01 (in blue for activation). (D) Co-IPs from S2 cells transfected with MYC-dpCLK and FLAG-dpPER expressing plasmids (500 ng each) without or with dsRNA targeting *dHsp68* 5' and 3' UTRs (15 μg each) and with either wild-type or mutant R45A FLAG-tagged dHSP68 (500 ng each). Co-IPs were probed with anti-MYC antibody, and WBs were performed using the indicated antibodies. (E) Co-IPs from S2 cells transfected with dsRNA against the 5' and 3' UTRs of both *dTrx* and *dHsp68* (15 μg each); plasmids expressing MYC-dpCLK, FLAG-dpPER, or dTRX-CT-V5 (500 ng each); and either wild-type or mutant R45A FLAG-tagged dHSP68 (400 ng each). Co-IPs were probed with anti-MYC antibody, and WBs were performed using the indicated antibodies. (F) Co-IPs from S2 cells transfected with dsRNA against *dHsp68* 5' and 3' UTRs (15 μg each) and plasmids expressing either dpCLK-V5 (500 ng) or dpCLKΔ19r-V5 (500 ng) with either wild-type or mutant R45A FLAG-tagged dHSP68 (400 ng each). Co-IPs were probed with anti-FLAG antibody, and WBs were performed using the indicated antibodies. Asterisks: black, dHSP68; red, dHSP68R45A. (G) Co-IPs from S2 cells transfected with dsRNA against *dTrx* 5' and 3' UTRs and plasmids expressing dTRX-CT-V5 (500 ng) and either MYC-dpCLK (500 ng) or MYC-dpCLKΔ19r (500 ng) in the presence or absence of dsRNA against *dHsp68* 5' and 3' UTRs (15 μg each). Co-IPs were probed with anti-MYC antibody, and WBs were performed using the indicated antibodies. Asterisks: black, dpCLK; red, dpCLKΔ19r. (H) Co-IPs from S2 cells transfected with FLAG-dpPER (500 ng), dsRNA against *dHsp68* 5' and 3' UTRs (15 μg each), and either wild-type or mutant R45A V5-tagged dHSP68 (500 ng each). Co-IPs were probed with anti-FLAG antibody, and WBs were performed using the indicated antibodies. Asterisks: black, dHSP68; red, dHSP68R45A. IP, immunoprecipitation.



**Fig. 5.** The repressive function of methylated HSP68 on R45 is conserved in light-driven monarch DpN1 cell rhythms. (A) Bioluminescence recordings from dpPER::LUC DpN1 cells in which repression by the major repressor CRY2 was attenuated via RNAi knockdown of endogenous *Bmal1* (*Bmal1i*), overexpression of a *Bmal1ΔCter* mutant lacking an  $\alpha$ -helix involved in CRY2 repression (*Bmal1Δ*), and knockdown of endogenous *Cry2* (*Cry2i*; hereafter called sensitized cells) in the presence of either dsRNA against *Trx* (*Trxi*) or increasing doses of dsRNA against *Hsp68s* orthologs (*Hsp68s 1Xi* and *2Xi*). RNAi knockdown of eGFP (*eGFPi*) was used as a control. Values are represented by mean  $\pm$  SEM of three replicates. (B) Quantification of dpPER::LUC bioluminescence from A at troughs and peaks of days 2 and 3 posttransfection. One-way ANOVA, Tukey post hoc:  $**P < 0.01$ . (C) Bioluminescence recordings from sensitized dpPER::LUC DpN1 cells in the presence of increasing doses of dsRNA against *Hsp68s* orthologs without or with overexpression of wild-type or R45A mutant dHSP68. (D) Quantification of dpPER::LUC bioluminescence from C at troughs and peaks of days 2 and 3 posttransfection. One-way ANOVA, Tukey post hoc:  $**P < 0.01$ , and ns is nonsignificant.

transcriptional activation that is independent of its methylated state and ability to bind to CLK and one that is dependent on its R45 methylation for PER repression, as suggested by the finding that PER and HSP68 interact in the absence of CLK in an R45 methylation-dependent manner (Fig. 4H).

**Methylated HSP68 on R45 Functions in Repression in Light-Driven Monarch DpN1 Cell Rhythms.** To confirm the repressive function of HSP68 in the monarch system, where circadian repression of CLK:BMAL1-dependent transactivation is mediated by PER and CRY2, we constructed a stable dpPER::LUCIFERASE (dpPER::LUC) reporter in the monarch DpN1 cell line by knocking in *luciferase* in frame with the 3' end of endogenous *per* using CRISPR-Cas9-mediated homology-directed repair (Fig. 5). The resulting cell line exhibited PER::LUC rhythms in

phase with those of endogenous PER (13) (Fig. 5A) in light-dark cycles, demonstrating proper functionality of the fusion dpPER::LUC protein. Of note, this cell line does not cycle under constant dark conditions (DD), and thus, circadian rhythms could not be observed in DD.

Because CRY2 acts as the major circadian repressor, we reasoned that weakening repression by CRY2 may be necessary to reveal the effects of HSP68 levels on PER repression, as these could be otherwise masked in the presence of a potent repression by CRY2. We thus sought to decrease CRY2 repression without completely abrogating the dpPER::LUC rhythms to uncover effects of *Hsp68* knockdown on PER repression. To this end, we knocked down endogenous *Bmal1* and overexpressed a *Bmal1* variant lacking the C-terminal  $\alpha$ -helix necessary for CRY2 repressive action [*Bmal1ΔCter* (15)] without or



with additional *Cry2* knockdown. As expected, knocking down *Bmal1* significantly decreased dpPER::LUC activation levels, and overexpression of *Bmal1* $\Delta$ *Cter* increased activation, which was further elevated by knockdown of *Cry2*, albeit to a significantly lesser level than in control cells (Fig. 5 *A* and *B*). In these sensitized cells, we confirmed the role of TRX in clock function by showing that additional knockdown of monarch *Trx* led to a significant decrease in activation levels at both trough and peak of the rhythm (Fig. 5 *A* and *B*). Consistent with our hypothesis that HSP68 is involved in repression, we also demonstrated that increasing doses of dsRNA against the three endogenous *Hsp68* orthologs led to a dose-dependent increase in activation levels (Fig. 5 *A* and *B* and *SI Appendix*, Fig. S4). Importantly, overexpression of *Drosophila* HSP68 in sensitized DpN1 cells with the lowest dose of dsRNA against endogenous *Hsp68s* restored low activation levels, while overexpression of HSP68R45A has no effect (Fig. 5 *C* and *D*), demonstrating that the critical role of methylation of R45 on HSP68 for PER repression initially observed in S2 cells is conserved in monarch DpN1 cells.

## Discussion

Circadian repression, which relies in most animals on the interaction of PER and CRY complexes with circadian activators, is central to the generation of 24-h rhythms. While the mechanisms by which CRY exerts its transcriptional repressive function are increasingly understood, the modalities of PER repressive action are still unclear. To date, the most compelling mechanistic evidence comes from studies in *Drosophila* and in mammalian cells, which demonstrated that PER represses by displacing CLK:BMAL1 from DNA (23–26). In *Drosophila*, PER represses in two phases, first by binding the activator complex on DNA and then by sequestering it off DNA (23). In mammals, repression also occurs in two consecutive phases, with a displacement-type repression mediated by PER–CRY followed by a blocking-type repression independent of PER (24–26). How PER initiates repression has remained an open question. Our study in the monarch butterfly uncovers the histone methyltransferase TRX and HSP68 as key molecules required for PER repression and shows that TRX is not only necessary for CLK:BMAL1-mediated transcriptional activation but is also crucial for PER repression via a previously unknown TRX-dependent arginine methylation of HSP68. Based on our data, we propose a model where the catalytically active TRX binds to CLK via the CLKe19r domain to promote CLK:BMAL1-mediated transcription. TRX then enzymatically contributes to the methylation of HSP68 on arginine 45 either directly or indirectly by promoting the activity of a yet unknown PRMT that methylates HSP68. Binding of methylated HSP68 on R45 to the CLK–TRX complex ultimately permits the recruitment of PER on the activator complex to initiate repression (Fig. 6).

Although our model is not based on interactions of chromatin-bound proteins, our functional luciferase transcriptional assays support the idea that it likely reflects events that occur on DNA. Consistent with the finding that mammalian MLL1 binds to CLOCKe19 on DNA (10), we showed that TRX binds to CLK through CLKe19r and contributes to transcriptional activation, suggesting that the function of TRX/MLL1 is

conserved within animal clocks. However, in our study, the catalytic function of TRX seems to primarily affect CLK:BMAL1 repression by PER rather than transcriptional activation as previously shown in mammals (10), as both  $\Delta$ SET and N3665A TRX catalytically inactive mutants restore activation to wild-type levels in luciferase assays. Although our data unambiguously show that TRX catalytic function is necessary for the methylation of HSP68 on R45, whether TRX acts directly or indirectly remains to be determined. The ability of TRX to directly methylate arginines remains a formal possibility, but it seems rather unlikely as no evidence exists, to our knowledge, to that effect. Instead, TRX may be involved in the recruitment of a PRMT. The fact that *Drosophila* Ash2L, a component of the multiprotein complex that assembles to TRX/MLL1 for its catalytic function (17, 18), is itself methylated at a single arginine residue by PRMT1 (27) supports this idea.

Regardless of the nature of the protein responsible for methylating HSP68, our finding that HSP68 methylation on R45 is required for transcriptional repression by PER raises the intriguing possibility that arginine methylation of HSPs could play an important role in the regulation of gene transcription. The impact of HSP methylation in the regulation of gene transcription is not without precedent, as methylation of another highly conserved arginine residue at position 469 of HSP70, the mammalian homolog of HSP68, has been shown to modulate the recruitment of a key component in the preinitiation complex and thus, transcription initiation (22). Our findings that methylation of HSP68 R45, which is a conserved residue across species, is necessary for binding to a transcriptional repressor favor the idea that HSP methylation may have a broader role in the regulation of gene transcription than previously assumed. In the context of time-keeping mechanisms by the circadian clock, the discovery that HSP68 plays a critical role in PER repression within the monarch clockwork extends the possible key functions of HSPs in circadian clocks. In plants, the adenosine triphosphate (ATP)-dependent molecular chaperone HSP90, in complex with HSP70 and HSP70/HSP90 organizing protein, functions within the core oscillator by facilitating the folding and maturation of one of its target proteins, the F box–type E3 ubiquitin ligase ZEITLUPE (28). Similarly, in mammals, HSP90 stabilizes BMAL1 through its ATP-dependent chaperone activity (29, 30). Given that proteins comprising the negative arm of the circadian clock network, including PERs from humans, mice, and insects, display a significant amount of predicted sequence disorder (31, 32), it is tempting to speculate that HSPs could also function to ensure the proper folding of PER for its binding to CLK to function as a circadian repressor. Whether this is the case and which role, if any, methylation of R45 on HSP68 plays in this process warrant further studies. Determining whether HSP68's function in PER repression is conserved in other species will also be important.

Finally, in mammals, CLKe19 has previously been shown to be required for cooperative binding of CLOCK:BMAL1 heterodimers to tandem E boxes (33), subsequent recruitment of MLL1 (10), and the binding of the repressor CLOCK-interacting protein circadian (CIPC) that occurs independently of PER (9, 34, 35). Our finding that CLKe19r is required for indirect PER binding to

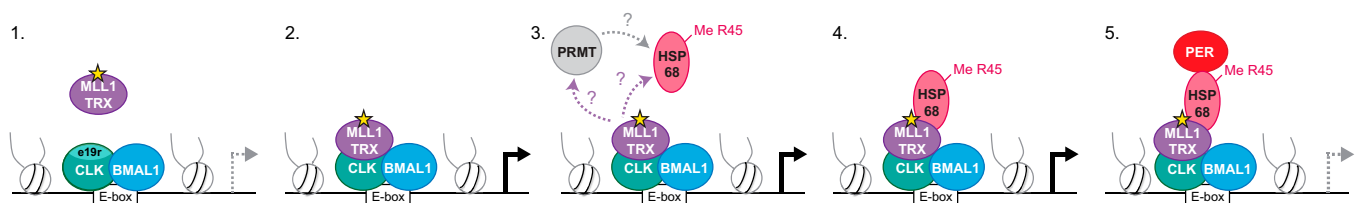


Fig. 6. Proposed model for TRX and HSP68 respective mode of action in activation and repression by PER within the clockwork.

CLK via HSP68 and for PER-mediated circadian repression adds further support to the emerging notion that the CLK<sup>E19r</sup> domain may act as a hub for the assembly of larger macromolecular complexes on DNA for both circadian activation and repression, as previously suggested (36). The parallel between the convergence on CLK<sup>E19</sup> for PER and CIPC repression and the convergence on the BMAL1 TAD for CRY and CHRONO repression (1, 3, 37–39) may suggest the existence of a greater number of repressive complexes acting coordinately during the repressive phase of the circadian cycle than previously envisioned.

## Materials and Methods

Detailed material and methods are provided in *SI Appendix, Materials and Methods*.

**Monarch Butterfly CLK<sup>Δ19r</sup> Mutant Line Generation and Husbandry.** Monarch CLK<sup>Δ19r</sup> mutants were generated using CRISPR-Cas9–targeted mutagenesis and maintained as described in *SI Appendix*.

**Eclosion Behavior.** Eclosion behavior assays were performed as described in ref. 15.

**S2 Cell Culture, Transfections, Transcriptional Assays, and Western Blotting.** Conditions of culture and transfections of *Drosophila* S2 cells, protein

extractions, procedures for luciferase assays, and western blotting, including antibodies and dilutions used, are described in *SI Appendix*.

**Real-Time qRT-PCR.** Quantification of circadian expression of *per* and *tim* in the monarch brain was performed as previously described (40). Procedures for quantification of *dTrx* and *dHsp68* levels in RNAi experiments in S2 cells, including RNA extraction and primers used, are described in *SI Appendix*.

**Co-IPs and Mass Spectrometry.** Conditions for protein extractions, antibodies/dilutions, and procedures used for co-IPs and mass spectrometry are described in *SI Appendix*.

**Monarch DpN1 PER::LUC Cells.** Procedures to insert a luciferase reporter in frame with *per* in monarch DpN1 cells using CRISPR-Cas9–assisted homology-directed repair and establish a stable cell line are described in *SI Appendix*.

**Data Availability.** All data are included in the manuscript and/or supporting information.

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